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Flavonoid biosynthesis genes in *Triticum aestivum* L.: methylation patterns in *cis*-regulatory regions of the duplicated *CHI* and *F3H* genes

Ksenia Strygina*, Elena Khlestkina

Federal Research Center N. I. Vavilov All-Russian Institute of Plant Genetic Resources, Russia
* Correspondence: k.strygina@vir.nw.ru

Abstract: Flavonoids ~~represent~~are a diverse group of ~~plant~~ secondary plant metabolites that play an important role in the regulation of plant development and ~~the~~ protection against ~~stress factors. Flavonoid~~ stressors. The biosynthesis of flavonoids occurs ~~by~~through the activity of several enzymes, including chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H). ~~Previously, a~~A functional divergence between some copies of the structural ~~genes~~ TaCHI and TaF3H ~~of~~genes was previously shown in the allohexaploid bread wheat *Triticum aestivum* L. (~~genome~~ BBAADD~~) was shown. Namely~~ genome). Specifically, *TaCHI-A1* and *TaCHI-D1* were strongly expressed in the coleoptile and roots, ~~but~~ while the expression of *TaCHI-B1* was ~~strongly expressed~~strong in the coleoptile and ~~weakly expressed~~weak in roots; *TaF3H-A1*, *TaF3H-B1*, and *TaF3H-D1* were expressed in different ~~parts of the~~ plant parts, except ~~for~~the roots, but *TaF3H-B2* was ~~specifically~~particularly expressed in the roots. We hypothesized that the ~~specificity~~specific nature of ~~the expression~~ *TaCHI* and *TaF3H* expression may be ~~caused~~induced by ~~promoters'~~the methylation~~.~~ of the promoter. It was found that the predicted position of CpG islands in the promoter regions of the analyzed genes and the actual location of methylation sites did not match. We found for the first time that ~~the~~ differences in the methylation status could affect ~~changes in~~ the expression of *TaCHI* copies, but not the expression~~, but not in~~ of *TaF3Hs*. At the same time, we revealed significant differences in the structure of the promoters of only the *TaF3H* genes, while the *TaCHI* promoters are highly homologous. We assume that the promoter structure in *TaF3Hs* primarily affects the change in the nature of gene expression. The data obtained are important for understanding the mechanisms ~~of~~that regulate the ~~formation of flavonoids~~ synthesis ~~features~~ of flavonoids in allopolyploid wheat and show that ~~the~~ differences in ~~promoters'~~the structure of promoters have a key effect on ~~the~~gene expression.

Keywords: ~~anthocyanins~~anthocyanin biosynthesis, ~~flavonoids~~flavonoid biosynthesis, duplicated genes, DNA methylation, promoter structure

~~**Citation:** Lastname, F.; Lastname, F.; Lastname, F. Title.~~**Citation:** Strygina,K.; Khlestkina, E. Flavonoid biosynthesis genes in *Triticum aestivum* L.: methylation patterns in *cis*-regulatory regions of the duplicated *CHI* and *F3H* genes. *Biomolecules* **2022**, *12*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

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1. Introduction

~~The regulation~~Regulation of gene expression is an important component ~~of~~in the ~~existence~~functioning of any organism. ~~Due to this a~~This implies constant control of various biological processes, such as growth, cell division, ~~environmental~~ response~~,~~ to environments or ~~stress, is performed.~~stressors, etc. Regulation of gene expression includes a wide range of mechanisms ~~used~~employed by cells to ~~change~~alter the expression level of specific genes and ~~to~~ change the number of gene products ~~of these genes~~ [1,2].

~~In~~Gene expression in multicellular organisms~~, the regulation of gene expression~~ is ~~based on~~regulated by genetic and epigenetic mechanisms. ~~The regulation~~Regulation occurs through a complex mechanism controlled by transcription factors (TFs) [3–5]. Epigenetic regulation is ~~carried out~~performed through modifications of DNA, RNA~~,~~ or

histone proteins [1,6,7]. An epigenetic factor, such as DNA methylation, is important for the binding of TFs to the regulatory region of genes.

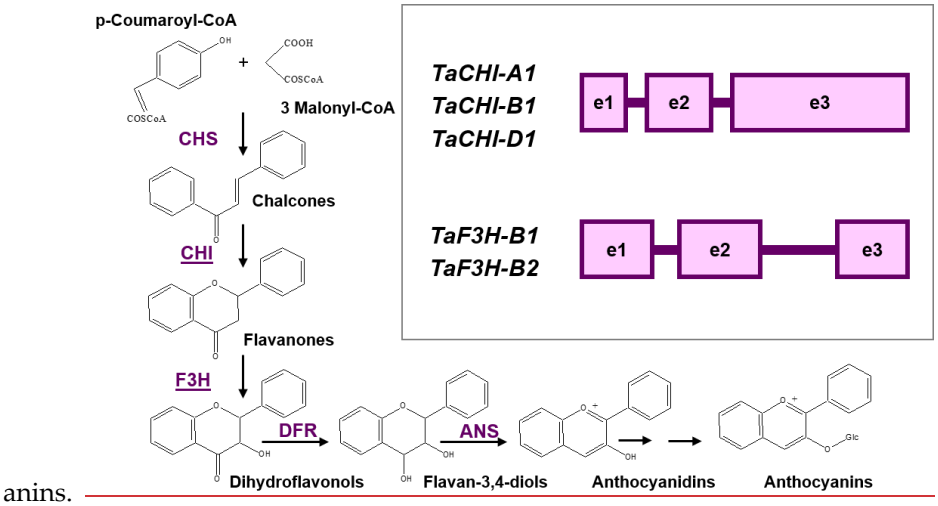
DNA methylation at cytosine residues is a covalent modification of cytosine (5-methylcytosine, 5mC), which is mainly found on the CpG dinucleotide in plants and animals. ~~However, for~~For plants, however, DNA methylation is observed not only at CpG sites~~,~~ but also at CpHpG and CpHpH sites, where H is adenine, cytosine~~,~~ or thymine [8,9]. Methylation of gene regions occurs more often at CpG sites, while non-CpG methylation (CpHpG and CpHpH) is much less common [10].

Cytosine methylation acts as a mechanism for controlling gene expression, which is often associated with long-term transcriptional repression [11]. Methylation in the 5'-region of the gene (including the promoter and part of the transcribed region) and the 3'-region (including part of the transcribed region and 3'-flanking sequences) could inhibit gene expression [6,12,13]. It has been suggested that methylation in the promoter region inhibits the binding of regulatory ~~protein~~proteins and, as a consequence, the transcription, while methylation within introns and exons correlates with highly expressed genes [13–15]. ~~In general~~Generally, cytosine methylation ~~provides~~makes a universal contribution to the growth and development of eukaryotic cells ~~by regulating~~through the regulation of gene activity.

Many important ~~crops~~crop species, including allohexaploid bread wheat (*Triticum aestivum* L., BBAADD genome, 2n = 6x = 42~~)~~), have a complex polyploid genome. ~~The regulation~~Regulation of gene expression in polyploid organisms, such as bread wheat, is complicated by the presence of ~~homoeologous~~homeologous copies in addition to paralogous ones [16]. This makes polyploid organisms an interesting model for studying ~~the~~ regulation of the expression of gene copies ~~expression~~, including ~~through~~the effect of DNA methylation [17–21].

Flavonoids, including anthocyanins ~~represent~~, are a diverse group of phenolic plant metabolites. Flavonoids act as plant pigments that ~~could color~~can stain tissues ~~in~~with various shades of reddish-purple, blue, and pink [22]. However, a significant proportion of flavonoid compounds ~~is~~are colorless.

Flavonoid biosynthesis is one of the most fully ~~characterized~~described metabolic pathways in plants [23,24]. ~~The biosynthesis~~Biosynthesis of the flavonoid compounds anthocyanidins, ~~the~~ precursors of anthocyanin pigments, requires the activity of seven enzymes: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3', 5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS, also known as LDOX~~—~~: leucoanthocyanidin reductase) (~~Fig.~~Figure 1). Enzymes belonging to the classes of methyltransferases (MT) and acyltransferases (AT) convert anthocyanidins ~~to~~into anthocy-



anins.

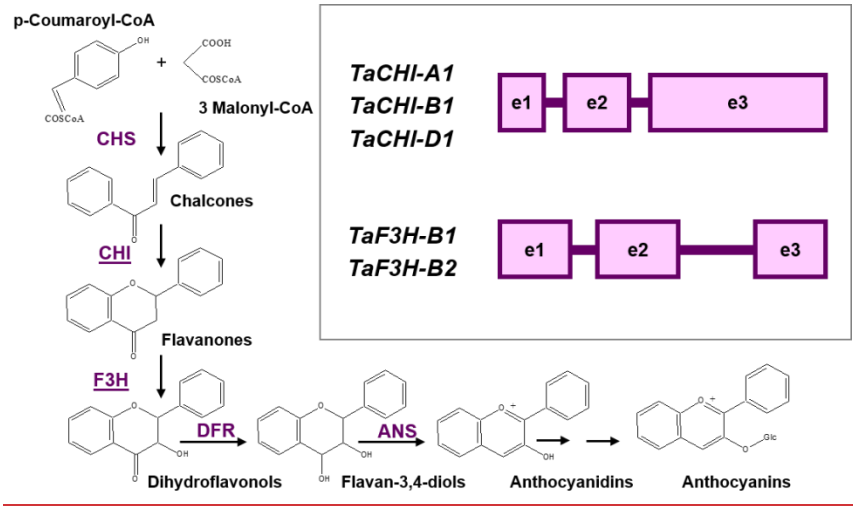


Figure 1. ~~The scheme~~Scheme of ~~anthocyanins~~the anthocyanin biosynthesis pathway. The rectangle shows the exon-~~intronic~~intron structure of the *TaCHI-A1*, *TaCHI-B1*~~,~~ and *TaCHI-D1*, ~~and~~plus *TaF3H-B1*~~,~~ and *TaF3H-B2* genes of bread wheat (*T.*-*aestivum*~~. The enzymes~~). Enzymes: ANS – anthocyanidin synthase; CHI – chalcone-flavanone isomerase; CHS – chalcone synthase; DFR – dihydroflavonol 4-reductase; F3H – flavanone 3-hydroxylase.

The sequences of key structural genes and their copies were identified in the flavonoid biosynthesis pathway ~~in~~within the *T. aestivum* genome~~ are identified~~ [25,26,35,27–34]. ~~We have previously shown~~Previously we showed the functional divergence between three *CHI* copies (*TaCHI-A1*, *TaCHI-B1*~~,~~ and *TaCHI-D1*) and four *F3H* copies (*TaF3H-A1*, *TaF3H-B1*, *TaF3H-B2*~~,~~ and *TaF3H-D1*) in *T.*-*aestivum* (~~Fig.~~Figure 1, 2) [29,30,32]. ~~By~~The RT-PCR ~~it was shown~~demonstrated that *TaCHI-A1* and *TaCHI-D1* ~~are~~were strongly expressed in the coleoptile and roots, ~~but~~while *TaCHI-B1* ~~is~~was strongly expressed in the coleoptile and weakly ~~expressed~~ in the roots. *TaF3H-B2* ~~is specifically~~was particularly expressed in wheat roots, unlike the *TaF3H-A1*, *TaF3H-B1*~~,~~ and *TaF3H-D1* genes~~, which are not expressed~~: their expression was absent in the roots but ~~are expressed~~occurred in various other ~~different~~plant parts~~ of the plant~~, including the coleoptile (~~Fig.~~Figure 2). The relative expression level of each copy in different wheat tissues ~~of wheat has~~was not ~~been~~ measured due to the high identity of gene sequences (copies ~~have~~had over 96-% identity) [30,32,36–40]. Nevertheless, it was shown that the total *TaCHI*s expression in shoots ~~is~~was higher than in roots, ~~and~~while the *TaF3H-1* genes ~~have~~had higher expression in the anthocyanin-colored coleoptile compared to ~~uncolored~~the colorless one, and the *TaF3H-1* and *TaCHI* expression in seedlings ~~increases during~~increased under salinity stress

[25,36,41]. However, the regulation mechanisms ~~of regulation of~~for tissue-specific expression of these gene copies ~~are still~~have been poorly studied.

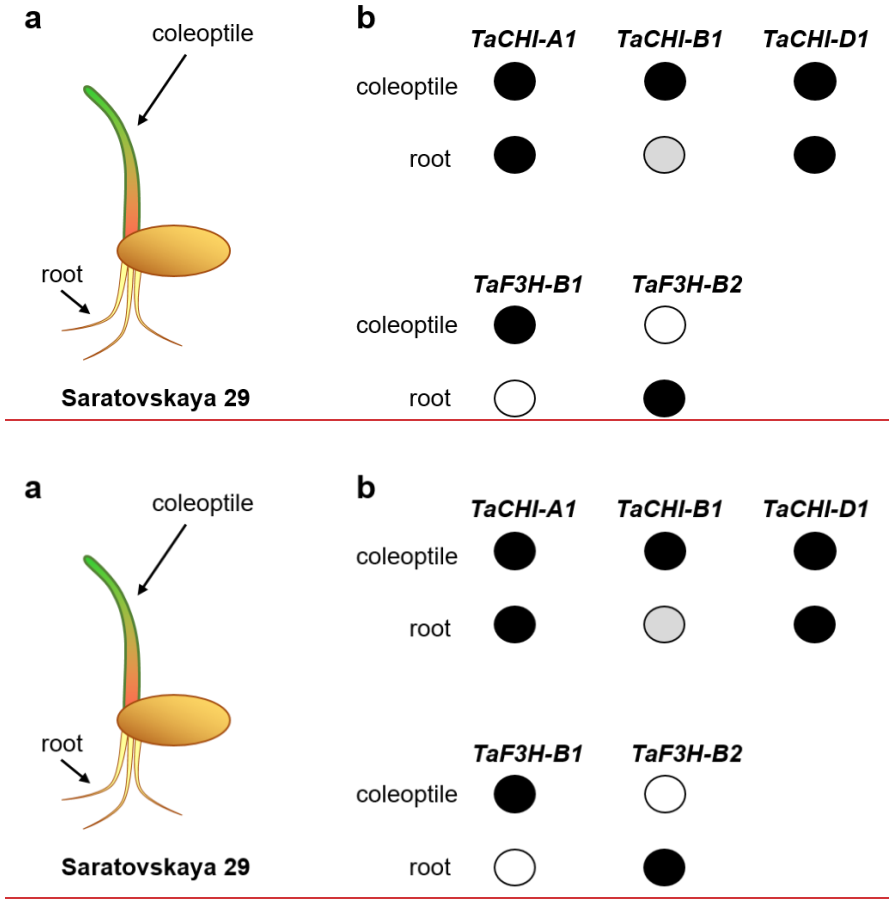


Figure 2. (a) ~~The~~ A germinating grain of ~~wheat's~~the wheat cultivar 'Saratovskaya ~~29~~29'. (b) ~~The expression~~Expression of the *TaCHI-A1*, *TaCHI-B1*~~,~~ and *TaCHI-D1*, ~~and~~plus *TaF3H-B1*~~,~~ and *TaF3H-B2* genes in 'Saratovskaya ~~29~~29'. The data concerning (1) *TaCHI*s expression ~~was~~were obtained ~~by~~using RT-PCR ~~in~~by Shoeva et al., (2014), and (2) *TaF3H*s expression ~~was~~were obtained ~~by~~using RT-PCR ~~in~~by Shoeva and Khlestkina, (2013). The black circle –means strong expression, the grey circle –means weak expression, and the white circle –means no expression.

~~In this work, we~~We hypothesized in this study that the ~~specificity~~specific nature of the expression ~~of~~manifested by individual copies of the flavonoid biosynthesis genes *TaCHI*s and *TaF3H*s may be related to the difference in methylation patterns of the same copies in different tissues. ~~To test the hypothesis, homoeologous~~The homeologous *TaCHI-A1*, *TaCHI-B1* and *TaCHI-D1*, and paralogous *TaF3H-B1* and *TaF3H-B2* genes were selected~~.~~ to test this hypothesis. We predicted possible sites of methylation and compared it with the actual location of 5mC in two organs of *T. aestivum*, in which a significant change in the expression level of *TaCHI*s and *TaF3H*s genes was previously shown. We also compared whether methylation coincides with the binding sites of key transcription factors. The results showed that differences in the methylation status could affect changes in ~~*TaCHI*s~~the expression of *TaCHI*s, but not ~~in~~ *TaF3H*s. We assume that ~~in in *TaF3H*s, the structure of~~ the promoter structure in *TaF3H*s primarily affects the change in the ~~character~~nature of gene expression. The obtained results provide ~~a~~ better understanding of the mechanisms ~~of~~underpinning tissue-specific regulation of gene expression in polyploid organisms.

2. Materials and Methods

Plant material, DNA isolation, and sodium bisulfite treatment

The plant material used in this study ~~includes~~included the allohexaploid wheat ~~cultivar (~~*T. aestivum*~~-)~~ cultivar 'Saratovskaya ~~29~~29'. To isolate DNA from the coleoptile and roots, 15 wheat seeds were germinated in a Rubarth Apparate ~~climatic~~climate chamber (RUMED ~~GmbH~~) on wet filter paper ~~at~~with a 12-hour photoperiod with LEDs plant growth (RUMED) at 20°C. Total genomic DNA was isolated from the coleoptile (weak anthocyanin coloration) and roots (~~lack~~absence of pigmentation) (**Fig. 2**) using the DNeasy Plant Mini Kit (QIAGEN) on the fifth day after germination. ~~Using the~~The EpiTect Fast Bisulfite Kit (QIAGEN~~), one~~) was used to treat 1 µg of the genomic DNA ~~of~~from each sample ~~was treated~~ with sodium bisulfite according to the manufacturer's instructions.

~~***Promoters'***~~***In silico promoter analysis, ~~primers~~and primers' design~~, polymerase chain reaction (PCR), PCR fragments separation, extraction, and*** ~~purification~~***

~~Alignment of~~The *TaCHI-A1* (GenBank: JN039037), *TaCHI-B1* (GenBank: JN039038), *TaCHI-D1* (GenBank: JN039039), *TaF3H-B1* (GenBank: AB223025), and *TaF3H-B2* (GenBank: JN384122) nucleotide sequences were ~~obtained by~~aligned using Clustal Omega and Multalin Multiple Sequence alignment [42,43]. The available promoter sequences were analyzed with New PLACE [44] and PlantPAN 3.0 [45–47]. ~~The~~ Prediction of CpG islands ~~prediction~~ and ~~the design of the primers~~primer designing were ~~performed~~carried out using MethPrimer 2.0 and Oligo 7 Primer Analysis Software [48,49]. The primers were designed against cytosine-converted sequences. The sequences of primers used in the study are ~~given~~listed in Table 1.

PCR, electrophoretic analysis, extraction, and purification

DNA amplification was ~~carried out~~performed in 20 µl of PCR mixture with 70 ng of the DNA template, 1 ng of each primer, and HotStarTaq DNA Polymerase (QIAGEN) according to the manufacturer's instructions. After initial denaturation at 94°C for 15 min, 35 cycles were ~~carried out~~implemented at 94°C for 1 min, 50–~~–~~55°C for 1 min, and 72°C for 2 min, followed by a final elongation step at 72°C for 5 minutes. Electrophoretic analysis was performed in 1% agarose gel (HydraGene Co.) prepared on a TAE buffer (40 mM Tris-HCl, pH 8.0; 20 mM sodium acetate; 1 mM EDTA) with ethidium bromide. Amplified fragments were isolated from agarose gel using the MinElute Gel Extraction Kit (QIAGEN).

Cloning and sequencing the amplified PCR fragments

The amplified PCR products were cloned for each sample ~~were cloned~~ using the PCR Cloning Kit (QIAGEN) and *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 XL-1 Blue competent cells (Evrogen). ~~Isolation of plasmid~~Plasmid DNA was ~~performed~~isolated using the ~~«~~"diaGene~~»~~" kit (DIA-M) according to the manufacturer's instructions. Plasmid DNA of 10 positive clones for each PCR product was amplified in both directions using M13 primers. DNA sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) and the SB RAS Genomics core facilities (Novosibirsk, Russia). All sequences obtained were deposited in GenBank (NCBI): *TaCHI-A1-coleoptile* MZ826579-MZ826588, *TaCHI-A1-root* MZ826589-MZ826598, *TaCHI-B1-coleoptile* MZ826599-MZ826608, *TaCHI-B1-root* MZ826609-MZ826618, *TaCHI-D1-coleoptile* MZ826619-MZ826628, *TaCHI-D1-root* MZ826629-MZ826638, *TaF3H-B1-coleoptile* MZ826639-MZ826648, *TaF3H-B1-root* MZ826649-MZ826658, *TaF3H-B2-coleoptile* MZ826659-MZ826668, and *TaF3H-B2-root* MZ826669-MZ826678.

3. Results

3.1. Prediction of CpG islands

CpG islands are DNA regions ~~that contain~~with a high frequency of CpG dinucleotides. We predicted possible sites of CpG methylation for the *TaCHI* and *TaF3H* genes *in silico* (Supplementary materials). The sequences of ~~the~~ *TaCHI* genes with promoters were used for the analysis (*TaCHI-A1* JN039037, *TaCHI-B1* JN039038, and *TaCHI-D1* JN039039). The gene promoters were aligned and compared (Supplementary materials). The ana-

lyzed regions ~~were about~~approximated 1600 bp~~, of which;~~ about 600 bp of them were ~~promoters'~~highly homologous promoter sequences. The presence of two islands was predicted for all *TaCHI*s. In the *TaCHI-A1* gene, Island 1 was in the promoter, and Island 2 ~~captures~~captured the end of the promoter, the ~~1st~~first exons, and the middle of the 1st intron. ~~In contradistinction to~~Unlike *TaCHI-A1*, Island 1 in the *TaCHI-B1* and *TaCHI-D1* genes~~, Island 1 ends~~ ended at the 1st exon and Island 2 ~~begins~~started on the 1st intron (Supplementary materials).

The promoters and exons of the *TaF3H* genes (*TaF3H-B1* AB223025, and *TaF3H-B2* JN384122) ~~differ~~differed in length and structure [29]. Thus, the length of the analyzed regions in *TaF3H-B1* and *TaF3H-B2* ~~genes~~ also differed. We used ~~for the analysis~~ the gene sequences with promoters for the analysis, and we found four CpG islands in *TaF3H-B1* and three ~~CpG islands~~ in *TaF3H-B2* (Supplementary materials). ~~In the~~Island 1 in *TaF3H-B1* ~~gene, Island 1 starts~~started in the middle of the promoter, while the other three islands ~~are~~were in the gene body. ~~In the~~Island 1 in *TaF3H-B2* ~~gene, Island 1 starts~~started 15 bp before the 1st exon; ~~Island~~Islands 2 and 3 ~~correspond~~corresponded to ~~Island~~Islands 3 and 4 ~~of a~~in *TaF3H-B1* ~~gene~~, respectively. The difference in the number of islands ~~is the reason for~~caused the differences in the structure of promoters (Supplementary materials).

3.2. ~~*Promoters' analysis*~~*Analysis of promoters*

~~Besides~~In addition to the basic motifs, like CAAT and CGCG boxes, the analysis of ~~the~~ promoter elements for the *TaCHI* and *TaF3H* genes revealed motifs responsible for light-dependent activation (yellow~~),~~) as well as TF-dependent elements required for genes involved in the biosynthesis of flavonoid compounds (red), tissue-specific (green~~),~~) and stress-specific elements (blue) (Supplementary materials). The most ~~represented~~representative elements were DOF, MYB, and MYC/bHLH. Among the light-dependent elements, only the *TaCHI* genes had SORLIP motifs (Sequences Over-Represented in Light-Induced Promoters). Among the stress-specific elements, the motifs for dehydration response ~~are~~were most typical for the *TaCHI* genes, and the reaction to heavy metals ~~—~~for ~~the~~ *TaF3H-B2* ~~gene~~. The *TaF3H-B2* promoter also ~~stands~~stood out ~~by~~for the abundance of pollen-specific motifs. The *TaCHI* genes ~~have~~had multiple root-specific motifs (Supplementary materials).

3.3. DNA methylation in the promoters of the TaCHI and TaF3H genes

~~To~~We developed specific primers for the promoters of each analyzed gene (Table 1) in order to verify the presence of CpG islands in the *cis*-regulatory regions and ~~to~~ identify ~~the~~ differences in methylation of regulatory elements~~, we developed specific primers to the promoters of each of the analyzed genes (Table 1).~~. The *TaCHI-D1* gene, ~~which has strong expression in~~strongly expressed in the coleoptile and roots, was completely unmethylated both in the coleoptile and in the roots (Figure ~~3c~~3, Table 2). In the *TaCHI-A1* gene (Table 2), ~~which is~~ strongly expressed in the coleoptile and roots, we detected large amounts of individual DNA methylation marks both at the CpG and non-CpG sites from the beginning of Island 2 to the beginning of the 1st exon (Figure ~~3a~~3, Supplementary materials). ~~In opposite~~Contrariwise, in the *TaCHI-B1* gene (Table 2), ~~which is~~ strongly expressed in the coleoptile and weakly ~~expressed~~ in the ~~root~~roots, we ~~detected~~found stable DNA methylation marks in the roots, but not in the coleoptile, at the beginning of the analyzed ~~area~~region (Figure ~~3b~~3). The marks ~~do~~did not correspond to the predicted islands (Supplementary materials). ~~Detected methylation~~ Methylation detected in both *TaCHI-A1* and *TaCHI-B1* ~~affects~~affected mostly the MYB ~~TFs~~TF recognition sites, light-induced elements, and stress-responsive ~~elements~~ones (Table 3).

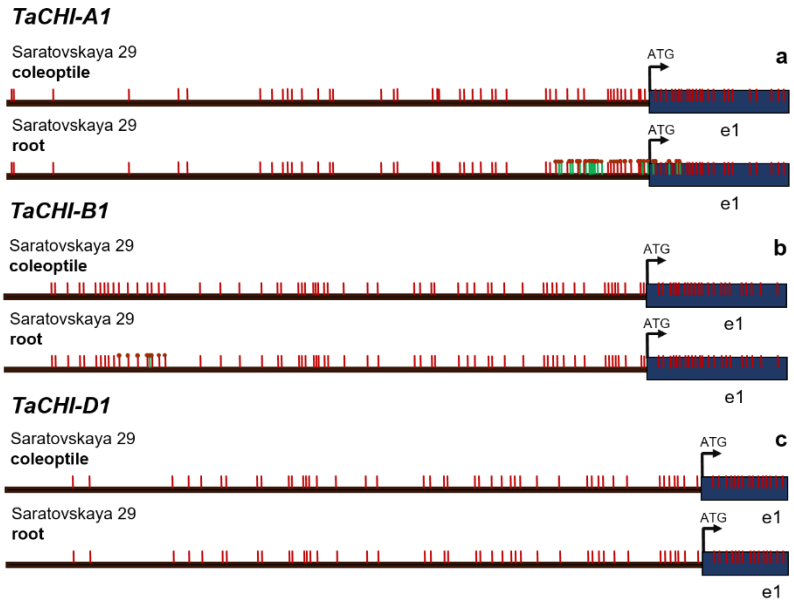


Figure 3. Methylation patterns of promoter regions ~~of~~for the (a) *TaCHI-A1* gene (799 bp), (b) *TaCHI-B1* (923 bp), and (c) *TaCHI-D1* (715 bp) in the wheat ~~in~~ coleoptile and ~~root~~roots. Arrows indicate the primers. Vertical red strokes are the predicted methylation sites. Vertical green ~~streaks~~strokes are the detected non-canonical methylation sites ~~detected~~. Red circles ~~are~~mark the detected methylation sites ~~detected~~.

Table 1. Gene-specific primers ~~that were~~ used for amplification of the *TaCHI* and *TaF3H* genes in the ~~current~~present study. R – purine (A, G), Y – pyrimidine (C, T)

Gene	Forward primer	Reverse primer	PCR product length (bp)	Annealing temperature~~.~~ (ºC)
TaCHI-A1	5′ TTATTAATTAAGTAGAAAAGAATTGTTTAGTTAA 3′	5′ TAAAAACRTAATTAATAATAATTAAAAAAAACAC 3′	374	50
	5′ GTTTTTGYGTGAGTTGAATGGTAG 3′	5′ ACRAAATAATCAACTAAAATAATAATACC 3′	298	50
	5′ GGAGAGGGTATTATTATTTTAGTTG 3′	5′ AAACCRTTAACTAAATAATCACCTAC 3′	273	50
TaCHI-B1	5′ TTTGATTATTGTTATTTTTATTTTAAATATGTATGT 3′	5′ ATATAAAAACRTAATTAATAATAATTAAAAAAAACA 3′	436	50
	5′ TYGTAGGGTGGTTTTTTTTGAGA 3′	5′ TAACRAACAAACACTTATATTAATAAAAC 3′	464	50
	5′ AGTTGTTGYGGTGTTATATAGGG 3′	5′ ACCAAATACRTTAAAACAAAATCTAAAATC 3′	343	53
TaCHI-D1	5′ TATATTTTATAAGGTGGTTTTTTTAATTTTGTTGAG 3′	5′ TAAAAACRTAATTAATAATAATTAAAAAAAAACAC 3′	320	53
	5′ GTGYGTGAGTTGAATGGTAGTTTG 3′	5′ TAATAATACRTACCCCCCCTATATAACACC 3′	294	55
	5′ GYGTAGGTTAGAGAATTAGATTAG 3′	5′ AAATAAACRTACTCRAAACCCRAC 3′	326	50
TaF3H-B1	5′ ATGATGTATAGGTTTTAGATATTGGG 3′	5′ ATATACRCACAACACACACATCAC 3′	400	53
	5′ ATATGAGYGTTTGTATTTGGATTGTG 3′	5′ CTCRTATAATAATTTATTCCTTAATAAAAAACTC 3′	365	53
	5′ TAAAYGGTYGAGTTTTTTATTAAGGAATAAATTATT 3′	5′ TCRAAAAAAACRTCTCGTTACTCACC 3′	125	53
TaF3H-B2	5′ GYGAGTTTAGATGGTTAGATATTTTTTGT 3′	5′ CACTAAATAAACATCACCAAAAAATCTAAAAT 3’	410	53
	5′ TTATTTTAGATTTTTTGGTGATGTTTATTTA 3′	5′ AAACAAAAACATCCTATTACTCAC 3’	222	50

Table 2. The number of methylation sites in the *TaCHI* and *TaF3H* genes. The table shows the ratio of the number of the identified methylated sites ~~that was identified~~ to the total number of ~~the~~ potential CpG, CpHpG, and CpHpH sites in the sequenced *E. coli* colonies

Gene	Tissue	Methylation sites in colonies		
		CpG	**CpHpG**	**CpHpH**
TaCHI-A1	coleoptile	0/60 in 10	0/54 in 10	0/125 in 10
	root	0/60 in 8 11/60 in 2	0/54 in 8 7/54 in 2	0/125 in 8 15/125 in 2
TaCHI-B1	coleoptile	0/50 in 10	0/42 in 10	0/118 in 10
	root	7/50 in 10	2/42 in 10	1/118 in 10
TaCHI-D1	coleoptile	0/44 in 10	0/46 in 10	0/112 in 10
	root	0/44 in 10	0/46 in 10	0/112 in 10
TaF3H-B1	coleoptile	25/59 in 4 26/59 in 6	4/41 in 4 5/41 in 1 7/41 in 4 8/41 in 1	12/72 in 2 13/72 in 1 14/72 in 1 18/72 in 1 25/72 in 2 26/72 in 2 27/72 in 1
	root	25/59 in 4 26/59 in 6	5/41 in 2 7/41 in 8	17/72 in 1 18/72 in 1 24/72 in 3 25/72 in 5
TaF3H-B2	coleoptile	0/12 in 6 1/12 in 1 3/12 in 1 4/12 in 3	0/13 in 6 2/13 in 4	0/76 in 6 3/76 in 1 4/76 in 3
	root	0/12 in 7	0/13 in 7	0/76 in 7

Gene	**Tissue**	**Methylation sites in colonies**		
		CpG	**CpHpG**	**CpHpH**
		4/12 in 3	2/13 in 3	6/76 in 3

241

242

Table 3. Methylation status of regulatory motifs in the *TaCHI*s and *TaF3H*s promoter sequences. R= = A/G, Y= = C/T, K= = G/T, W= = T/A, V= = A/C/G, B= = G/T/C, N= = A/T/G/C

					Methylation status	
Gene	**Motif name**	**Sequence**	**Function**	**Reference**	**Coleoptile**	**Root**
TaCHI-A1	ABRELATERD1	ACGTG	Element required for etiolation-induced expression of the gene responsive to dehydration	S000414	-	+
	ACGTATERD1	ACGT	Sequence required for etiolation-induced expression	S000415	-	+
	CBFHV	RYCGAC	Dehydration-responsive element	S000497	-	+
	CGACGOSAMY3	CGACG	Coupling element for the G box element	S000205	-	+
	CGCGBOXAT	VCGCGB	Element in promoters of many genes	S000501	-	+
	CRTDREHVCBF2	GTCGAC	Core CRT/DRE motif	S000411	-	+
	HEXAMERATH4	CCGTCG	Hexamer motif of the *Arabidopsis thaliana* histone H4 promoter	S000146	-	+
	MYBCORE	CNGTTR	Binding site for animal and plant MYB proteins	S000176	-	+
	SORLIP2AT	GGGCC	Sequences ~~Over Represented~~over-represented in ~~Light-Induced Promoters~~light-induced promoters	S000483	-	+
	SURECOREATSULTR11	GAGAC	Core of sulfur-responsive element	S000499	-	+
TaCHI-B1	CBFHV	RYCGAC	Dehydration-responsive element	S000497	-	+
	DRECRTCOREAT	RCCGAC	Core motif of a dehydration-responsive element~~/~~ / C-repeat *cis*-acting element	S000418	-	+
	LTRECOREATCOR15	CCGAC	Core of low temperature-responsive element	S000153	-	+
	MYB2CONSENSUSAT	YAACKG	MYB recognition site	S000409	-	+
	MYBCORE	CNGTTR	Binding site for animal and plant MYB proteins	S000176	-	+
	MYBCOREATCYCB1	AACGG	Myb core	S000502	-	+
	PALBOXAPC	CCGTCC	Box A	S000137	-	+
TaF3H-B1	ABRELATERD1	ACGTG	Element required for etiolation-induced expression of	S000414	+	+

					Methylation status	
Gene	**Motif name**	**Sequence**	**Function**	**Reference**	**Coleoptile**	**Root**
			the gene responsive to dehydration			
	ABRERATCAL	MACGYGB	"Repeated sequence motifs" identified DE in the upstream regions of Ca(2+)-responsive upregulated DE genes	S000507	+	+
	ARFAT	TGTCTC	ARF (auxin response factor) binding site found in the promoters DE of primary/early auxin response genes ~~of~~in *Arabidopsis thaliana*	S000270	+	+
	ACGTATERD1	ACGT	Sequence required for etiolation-induced expression	S000415	+	+
	CBFHV	RYCGAC	Dehydration-responsive element	S000497	+	+
	CGACGOSAMY3	CGACG	Coupling element for the G box element	S000205	+	+
	CGCGBOXAT	VCGCGB	Element in promoters of many genes	S000501	+	+
	CRTDREHVCBF2	GTCGAC	Core CRT/DRE motif	S000411	+	+
	CURECORECR	GTAC	Core of a copper-response element	S000493	+	+
	DOFCOREZM	AAAG	Core site required for binding of Dof	S000265	+	+
	DPBFCOREDCDC3	ACACNNG	Binding core sequence of f–bZIP transcription factors	S000292	+	+
	GATABOX	GATA	Motif required for high–level, light-regulated, and tissue-specific expression	S000039	+	+
	LTRE1HVBLT49	CCGAAA	Low-temperature-responsive element	S000250	+	+
	MYBCOREATCYCB1	AACGG	Myb core	S000502	+	+
	MYBST1	GGATA	Core motif of MYB binding site	S000180	+	+
	NODCON2GM	CTCTT	Putative nodulin consensus sequences	S000462	+	+
	OSE2ROOTNODULE	CTCTT	One of the consensus sequence motifs of organ-specific elements DE (OSE) characteristic of the promoters activated in infected cells DE of root nodules	S000468	+	+

					Methylation status	
Gene	**Motif name**	**Sequence**	**Function**	**Reference**	**Coleoptile**	**Root**
	RHERPATEXPA7	KCACGW	Right part of RHEs (Root Hair-specific *cis*-Elements)	S000512	+	+
	SEBFCONSSTPR10A	YTGTCWC	Binding site of the potato silencing element binding factor DE (SEBF) gene found in the promoter of the pathogenesis-related gene DE (PR-10a)	S000391	+	+
	SURECOREATSULTR11	GAGAC	Core of sulfur-responsive element	S000499	+	+
TaF3H-B2	CURECORECR	GTAC	Core motif of a CuRE (copper-response element)	S000493	+	+
	DPBFCOREDCDC3	ACACNNG	bZIP transcription factor binding core sequence	S000292	-	+
	ERELEE4	AWTTCAAA	Ethylene responsive element	S000037	+	-
	GATABOX	GATA	Motif required for high–level, light-regulated, and tissue-specific expression	S000039	+	+
	REALPHALGLHCB21	AACCAA	Motif required for phytochrome regulation; the DNA binding activity is high in etiolated plants	S000362	+	+
	RHERPATEXPA7	KCACGW	Right part of RHEs	S000512	-	+

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Analysis ofAnalyzing the methylation patterns ofin the *TaF3H* genes revealeddisclosed many methylation marks in Island 1 in the promoter of the *TaF3H-B1* gene (expressed in different plant parts of the plant, except for roots) and few marks out ofin Island 1 in the promoter of the *TaF3H-B2* gene (specificallyparticularly expressed in the roots) (Figure 4, Table 2). DetectedThe detected methylation affectsaffected mostly bZIP and MYB TFsTF recognition sites, and hormone- and stress-responsive elements (Table 3). However, we almost did not reveal theno differences were observed for both *TaF3H-B1* and *TaF3H-B2* in the DNA extracted from roots or coleoptiles for both *TaF3H-B1* and *TaF3H-B2*.

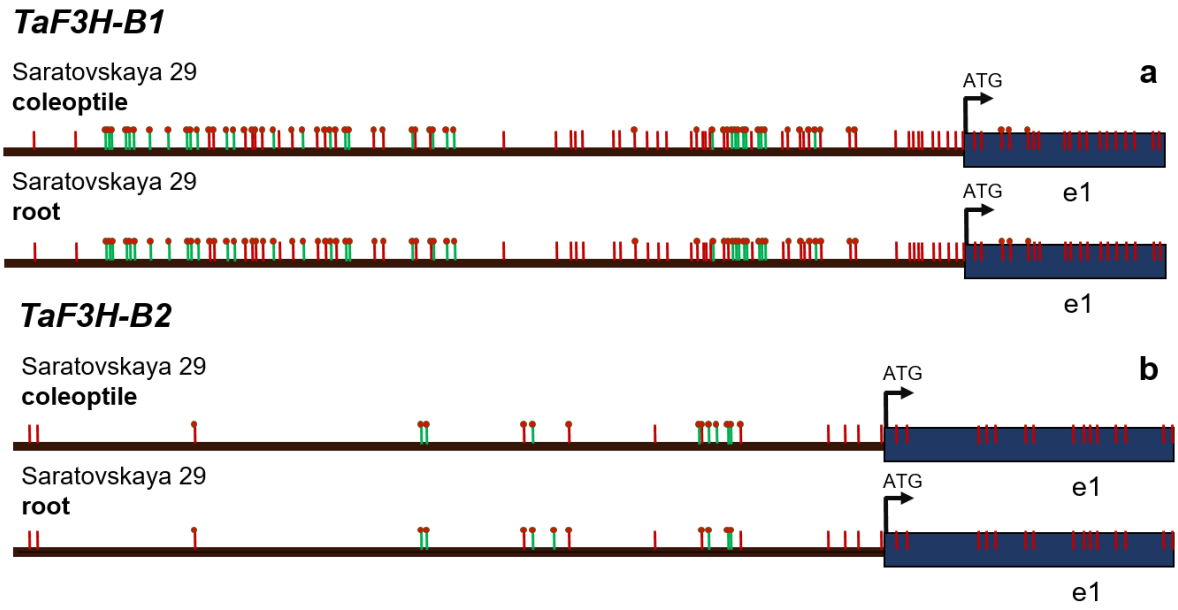


Figure 4. Methylation patterns of promoter regions offor the (a) *TaF3H-B1* gene (737 bp) and (b) *TaF3H-B2* (598 bp) ofgenes in the wheat in coleoptile and rootroots. Arrows indicate the primers. Vertical red strokes are the predicted methylation sites. Vertical green streaksstrokes are the detected non-canonical methylation sites detected. Red circles aremark the detected methylation sites detected.

4. Discussion

The implementation of biologicalBiological processes is carried outare implemented under the control of the spatial and temporal gene expression that is determined with high accuracy. Regulation of gene expression is an important aspect ofin the life of all organisms, therefore its adaptation in the context of evolution is especially significant [50–52]. Numerous sequential changes, including gene duplication, are a necessary part of thesuch adaptation. For example, duplication of genes encoding TFs, followed by the structural and functional divergence of the obtained copies, makes an essential contribution to the development of transcription regulatory networks development [17,52–55]. The redundancy provided by duplicated genes could contribute to the adaptation of species and genetic resistance to changes in environmental conditions [18]. Normalization of the gene dose occurs due to genetic and epigenetic changes. For example, among three homoeologoushomeologous copies of the *LEAFY HULL STERILE1* (*WLHS1*) gene of the allohexaploid bread wheat *T. aestivum*, one gene lost its functionality due to a mutation in the functional domain, while the other copy was not transcribed due to hypermethylation, and only the third gene retained its functionality [56]. In this workstudy, we decided to establishfind what iswas hidden behind the functional divergence of *TaCHI* and *TaF3H* gene copies ofin *T. aestivum*. We assumed that the reason iscause was the

tissue-specific gene expression associated with differences in the ~~promoter's~~promoter structure and ~~in~~ the methylation pattern.

Previously it was shown that *TaCHIs* ~~are~~were expressed in different parts of wheat independently on ~~its~~the coloration~~. Although~~, even though in some intensively colored organs ~~some~~a certain increase ~~of~~in the gene expression can be observed [30]. The promoter structure of *TaCHI-B1* ~~is~~was insignificantly different from that of *TaCHI-A1* ~~and~~or *TaCHI-D1*, with the presence of additional MYB-recognition elements and G-box (ACE~~—~~: ACGT-containing ~~region~~element) [30]. ~~It is likely that these~~These differences, together with the identified stable methylation marks, ~~were~~are likely to be the cause of ~~the~~a decreased gene expression in roots (Figure 3b, Additional file). ~~It is known that~~ MYB TFs are known to serve as key regulators ~~at~~for the synthesis of flavonoids in various plant organs [37,55,57–60]. Methylation ~~marks~~ in *TaCHI-B1* ~~were~~was stable and ~~were found~~appeared in all clones at the beginning of the analyzed region. We assume that this site may be critical for a decrease in the level of gene expression ~~particularly~~, especially because this ~~area~~region contains MYB-binding domains.

~~Based on the~~The results obtained for the *TaCHI-A1* gene~~,~~ implied that the presence of methylation marks in the promoter and the 1st exon in two clones did not affect the gene expression (Figure 3a). Nevertheless, ~~in~~ Shoeva et al. (2014b~~), it is noticeable~~) noticed that in ~~the cultivar~~ cv. 'Saratovskaya ~~29~~29' with weak anthocyanin pigmentation of the coleoptile (coincides with the genotype studied here~~),~~) the expression of *TaCHI-A1* ~~is~~was weaker than in the near-isogenic lines (NILs) i:S29Pp1Pp2 with strong anthocyanin pigmentation of the coleoptile. This difference could be explained by the presence ~~in the NILs~~ of the dominant ~~flavonoids~~flavonoid biosynthesis regulator gene *TaMyc-A1* (~~coding~~encoding MYC/bHLH TF~~),~~) in the NILs, which ~~leads~~led to a purple color change in many parts of the plant [61,62].

~~In wheat, 4~~Four copies of the gene encoding F3H were previously identified in wheat. Three copies (*TaF3H-A1*, *TaF3H-B1*, and *TaF3H-D1*) ~~were formed as a result of~~resulted from allopolyploidization of the wheat genome. These genes are co-transcribed and localized in the ~~synthenic~~syntenic regions of chromosomes 2A, 2B and 2D, ~~and show~~showing high similarity in the structure of the coding regions and promoters [25]. The fourth copy (*TaF3H-B2*) is a paralogous copy in chromosome 2B, differing in the structure and transcriptional activity [29].

The presence of a paralogous copy of *F3H* in wheat and in some of its relatives is unique among plants. Comparison ~~of~~between *TaF3H-B2* ~~with~~and *TaF3H-1* copies indicates that conservative amino acid residues are still present in *TaF3H-B2*~~, which~~; they are necessary for the formation of the enzyme's active sites ~~of the enzyme~~ [29,63]. However, the fact that *TaF3H-B2* is not transcribed during the biosynthesis of anthocyanins, but is transcribed in wheat roots, suggests that *TaF3H-B2* probably ~~participating~~participates in the biosynthesis of other flavonoid compounds (~~whereas~~while the *TaF3H-1* genes do exactly the opposite).

~~In~~ Khlestkina et al. (2013) and ~~in~~ the ~~current work,~~present study demonstrated a significant difference in the structure of *TaF3H-1* and *TaF3H-2* promoters ~~was shown. In~~. For *TaF3H-B1*, active methylation both in the coleoptile and ~~root~~roots mainly affected the TF and stress-responsive elements and did not affect MYB-binding sites near the ATG start codon (Figure 4, Additional file). Moreover, only one type of the light-dependent element GATABOX, required for high~~—~~level and tissue-specific gene expression, was methylated (Table 3). We assume that, since the remaining light-dependent elements and TF~~—~~binding sites were not methylated, the expression of *TaF3H-B1* is possible in the coleoptile under the studied conditions.

It was previously ~~noted~~reported that *TaF3H-1* ~~are~~was expressed only in colored tissues and ~~are~~ not expressed in ~~non-colored~~colorless ones, such as the uncolored pericarp or roots of 'Saratovskaya ~~29~~29' [64]. ~~The other~~Other structural genes, such as *TaCHS*, *TaCHI*, *TaDFR* or *TaANS* ~~are~~, were transcribed in the absence of anthocyanin pigments, but at ~~the~~a lower level than in ~~comparison with the~~ intensively colored tissues. The lack

of this gene's expression ~~of this gene~~ in the pericarp and ~~root coincides~~roots coincided with the pattern of *TaMyc-A1* expression, ~~which suggests~~suggesting that the *TaF3H-1* genes are the key structural ~~gene~~genes that ~~determines~~determine the origin of anthocyanin biosynthesis and the main targets of TF [62]. The features ~~concerning~~associated with the regulation of expression or substrate specificity of *TaF3H-B2* have not yet been studied. ~~Weak~~The weak methylation status in both ~~in~~the coleoptile and ~~root, and~~roots as well as single changes in the ~~promoters'~~promoter methylation pattern could not be the cause of the difference in *TaF3H-B2* activity (Figure 4, Table 3). According to the results ~~obtained, the~~ of this study, differences in ~~promoters'~~the promoter structure and TF specificity should lead to tissue-specific expression of these genes.

Thus, we ~~assumed~~assume that DNA methylation, ~~promoters'~~divergence of promoter sequences ~~divergence~~, and gene expression regulation together underpin the specific pattern of the duplicated *TaCHI* and *TaF3H* ~~duplicated~~ genes in *T. aestivum*.

Supplementary Materials: ~~The following supporting~~Supporting information can be downloaded ~~at~~from: www.mdpi.com/xxx/s1.

Author Contributions: KS performed all molecular-genetic and *in silico* experiments, contributed to data interpretation ~~of data,~~ and participated in the drafting of the manuscript. EK contributed to the conception and design of the study, ~~to~~data interpretation, and critical revision of ~~data and to revising~~ the manuscript ~~critically~~. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Russian Science Foundation ~~№~~, No. 21-66-00012.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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